

J. Timothy Meigs
Sector Patent Counsel

Novartis
Corporate Intellectual Property
9 W. Watkins Mill Road
Gaithersburg, MD 20878

Tel (301) 258-4715
Fax (301) 258-4620
tim.meigs@group.novartis.com



Fax

Attention Examiner Ruixiang Li

Fax no. 703-746-7186

Number of pages 10 including cover page

Date June 12, 2003

Concerning App. No. 09/817,487 – Reply After Final

Dear Examiner Li,

Attached for filing in the above application is a Reply After Final (4 pages), which also includes a copy of the postcard filing receipt from Applicant's previous submission, acknowledging receipt by the PTO on March 3, 2003, of the Declaration of Prior Invention including Exhibits A & B (1 page), as well as additional copies of the Declaration of Prior Invention under § 1.131 (4 pages).

Respectfully submitted,

A handwritten signature in black ink, appearing to read 'J. Timothy Meigs'.
J. Timothy Meigs
Attorney for Applicant
Registration No. 38,241

CONFIDENTIALITY NOTICE

THIS FAX CONTAINS CONFIDENTIAL INFORMATION WHICH MAY BE PRIVILEGED AND IS INTENDED SOLELY FOR THE USE OF THE ABOVE-NAMED RECIPIENT(S). IT MAY BE EXEMPT FROM DISCLOSURE UNDER APPLICABLE LAW. IF YOU ARE NOT AN INTENDED RECIPIENT OR PERSON RESPONSIBLE FOR DELIVERY OF THIS FAX TO AN INTENDED RECIPIENT, YOU ARE HEREBY NOTIFIED THAT YOU HAVE RECEIVED THIS FAX IN ERROR AND THAT ANY REVIEW, DISSEMINATION, DISCLOSURE, COPYING OR OTHER USE OF THIS FAX OR ITS CONTENTS IS STRICTLY PROHIBITED. IF YOU HAVE RECEIVED THIS FAX IN ERROR, PLEASE NOTIFY THE SENDER IMMEDIATELY BY TELEPHONE AT ONE OF THE PHONE NUMBERS SET FORTH ABOVE AND DESTROY THIS FAX IMMEDIATELY.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Pippig *et al.*

App. No. 09/817,487

Filed: March 26, 2001

For: Selectable Marker Genes

Confirmation No. 9170

Art Unit: 1646

Examiner: Ruixiang Li

Atty. Docket: 4-31193A

DECLARATION OF PRIOR INVENTION UNDER 37 C.F.R. § 1.131

U.S. Patent and Trademark Office
P.O. Box 2327
Arlington, VA 22202

Sir:

The inventors, Susanne Pippig and Gabor Veres, declare as follows:

1. We are former employees of Systemix, Inc., in Palo Alto, California. While employed at Systemix, we invented the invention described and claimed in U.S. Patent Application No. 09/539,248, filed March 30, 2000 (which was later converted to Provisional Application No. 60/266,331) and are therefore the named inventors thereof. We are also the named inventors of the above-captioned Application No. 09/817,487, which claims the benefit of Provisional Application No. 60/266,331.

2. It is our understanding that in a Non-Final Office Action dated Sept. 3, 2002, in Application No. 09/817,487, claims 1-4, 6-8, 10-12, and 21 were rejected under 35 U.S.C. § 102(a) as being anticipated by Zhou *et al.*, *J. Cell Biology* 146:1133-146 (September 6, 1999) and that claims 9, 14-20, 23, and 24 were rejected under 35 U.S.C. § 103(a) as being unpatentable over Zhou *et al.*, *J. Cell Biology* 146:1133-146 (September 6, 1999) in combination with one or more other references. We make this Declaration in a traversal of these rejections.

T:\Applications\31000's\31193A\Filed\31193A - 131 Declaration.doc

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	:	
	:	
Pippig <i>et al.</i>	:	Confirmation No. 9170
	:	
App. No. 09/817,487	:	Art Unit: 1646
	:	
Filed: March 26, 2001	:	Examiner: Ruixiang Li
	:	
For: Selectable Marker Genes	:	Atty Docket: 4-31193A

DECLARATION OF PRIOR INVENTION UNDER 37 C.F.R. § 1.131

U.S. Patent and Trademark Office
P.O. Box 2327
Arlington, VA 22202

Sir:

The inventors, Susanne Pippig and Gabor Veres, declare as follows:

1. We are former employees of Systemix, Inc., in Palo Alto, California. While employed at Systemix, we invented the invention described and claimed in U.S. Patent Application No. 09/539,248, filed March 30, 2000 (which was later converted to Provisional Application No. 60/266,331) and are therefore the named inventors thereof. We are also the named inventors of the above-captioned Application No. 09/817,487, which claims the benefit of Provisional Application No. 60/266,331.
2. It is our understanding that in a Non-Final Office Action dated Sept. 3, 2002, in Application No. 09/817,487, claims 1-4, 6-8, 10-12, and 21, were rejected under 35 U.S.C. § 102(a) as being anticipated by Zhou *et al.*, *J. Cell Biology* 146:1133-146 (September 6, 1999) and that claims 9, 14-20, 23, and 24 were rejected under 35 U.S.C. § 103(a) as being unpatentable over Zhou *et al.*, *J. Cell Biology* 146:1133-146 (September 6, 1999) in combination with one or more other references. We make this Declaration in a traversal of these rejections.

T:\Applications\31000\31193\4-31193A\Filed\31193A - 131 Declaration.doc

Pippig *et al.*
App. No. 09/817,487

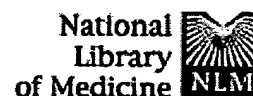
3. Prior to the September 6, 1999, publication date of the Zhou *et al.* paper, we conceived and reduced to practice the invention claimed in claims 1-9, 11, 12, 14, 15, 17-20, and 25-30 set forth in the accompanying Reply Under 37 C.F.R. § 1.111 responsive to the Office Action dated Sept. 3, 2002. As evidence of our prior invention, we attach hereto Exhibits A and B.
4. Exhibit A is a memorandum to us dated August 9, 1999, from Lynn Marcus-Wyner, the Systemix patent attorney who drafted parent Application No. 09/539,248, along with a draft of the application. This memorandum and draft application, particularly the "Experimental" section beginning on page 23, shows that prior to the publication date of the Zhou *et al.* paper, we conceived and reduced to practice the invention claimed in claims 1-9, 11, 12, 14, 15, 17-20, and 25-30 set forth in the accompanying Reply Under 37 C.F.R. § 1.111 responsive to the Office Action dated Sept. 3, 2002.
5. Exhibit B is an Affidavit of Lynn Marcus-Wyner, attesting to the authenticity of the memorandum and draft patent application provided as Exhibit A.
6. All statements made herein of our own knowledge are true and all statements made on information and belief are believed to be true. Further, these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Subscribed to on the following date:

Susanne Pippig, Ph.D.

02-24-2003

Gabor Veres, Ph.D.



PubMed

Nucleotide

Protein

Genome

Structure

PMC

Taxonomy

OMIM

Bo

Search PubMed

for

Limits

Preview/Index

History

Go Clear

Clipboard

Details

About Entrez

Display

Abstract

Show:

20

Sort

Send to

Text

Text Version

☐ 1: J Hematother 1996 Aug;5(4):323-9

Related Articles, Links

Entrez PubMed

Overview

Help | FAQ

Tutorial

New/Noteworthy

E-Utilities

PubMed Services

Journals Database

MeSH Database

Single Citation Matcher

Batch Citation Matcher

Clinical Queries

LinkOut

Cubby

Related Resources

Order Documents

NLM Gateway

TOXNET

Consumer Health

Clinical Alerts

ClinicalTrials.gov

PubMed Central

Privacy Policy

Improved retroviral vectors for hematopoietic stem cell protection and in vivo selection.

Baum C, Eckert HG, Stockschrader M, Just U, Hegewisch-Becker S, Hildinger M, Uhde A, John J, Ostertag W.

Abteilung Zell- und Virusgenetik, Heinrich-Pette-Institut für Experimentelle Virologie und Immunologie an der Universität Hamburg, Germany.

Therapeutic gene transfer into hematopoietic cells is critically dependent on the evolution of methods that allow ex vivo expansion, high-frequency transduction, and selection of gene-modified long-term repopulating cells. Progress in this area needs elaboration of defined culture and transduction conditions for long-term repopulating cells and improvement of gene transfer systems. We have optimized retroviral vector constructions based on murine leukemia viruses (MuLV) to overcome the transcriptional repression encountered with the use of conventional Moloney MuLV (MoMuLV) vectors in early hematopoietic progenitor cells (HPC). Novel retroviral vectors, termed FMEV (for Friend-MCF/MESV hybrid vectors), were cloned that mediate greatly improved gene expression in the myeloerythroid compartment. Transfer of the selectable marker multidrug resistance 1 (mdr1), FMEV, in contrast to conventional MoMuLV-related vectors currently in use for clinical protocols, mediated background-free selectability of transduced human HPC in the presence of myeloablative doses of the cytostatic agent paclitaxel in vitro. Furthermore, FMEV also greatly improved chemo-protection of hematopoietic progenitor cells in a murine model system in vivo. Finally, when a second gene was transferred along with mdr1 in an FMEV-backbone, close to 100% coexpression was observed in multidrug-resistant colonies. These observations have significant consequences for a number of ongoing and planned gene therapy trials, for example, stem cell protection to reduce the myelotoxic side effects of anticancer chemotherapy, correction of inherited disorders involving hematopoietic cells, and antagonism of HIV infection.

Publication Types:

- Review
- Review, Tutorial

09/817,487

09/817487

~~ CProject ~~

CProjectData

Selectable Marker Genes 4-31193A

CDNASequence

First Sequence

homo sapiens~f

atgagagagc	tgtcaacat	tccactggta	catattctta	ctctggttgc	cttcagcgga	60
actgagaaac	ttccaaaagc	tcctgtcatc	accactcctc	ttgaaacagt	ggatgcctta	120
gttgaagaag	tggctacttt	catgtgtgca	gtggaatcct	acccccagcc	tgagatttcc	180
tggactagaa	ataaaattct	cattaaactc	tttgacaccc	ggtacagcat	ccggggagaat	240

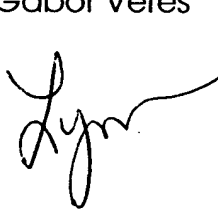
(sample of submitted file)



SyStemix, Inc.

MEMORANDUM

TO: Susanne Pippig and Gabor Veres

FROM: Lynn Marcus-Wyner 

CC: Melissa Shaw

DATE: August 9, 1999

RE: Draft Patent Specification
Docket No. 4-30922P1/USN
Entitled "Selectable Marker Genes"

I have enclosed a first draft for the MuSK-R patent application. As stated in the July 27th cover memo for the draft EGFR applkication, Melissa and I decided to file two separate applications.

Please read through the entire disclosure very carefully, and make a notation of any corrections, inaccuracies or questions that you may have. This can be done directly on the copy sent to you and then sent back to me.

I have provided, at numerous places in the disclosure, bold, italics and underlined sections. These sections are directed to questions, comments and other information still required or needing clarification. Please address each one of these sections. You can make notes or fill in blanks directly on the copy provided, but please write clearly.

Once you have had a chance to review the draft we should get together to go over this specification and the EGFR specification. I know Gabor is out of town until August 17th. I also will be out of town but back on August 24th. How about if we get together the morning of Friday August 27 or Monday August 30. It would not be necessary for Gabor to meet with us, but his comments are necessary. I think we should plan on meeting for at least 1.5 hours. It would be best if you could go through the specifications and provide reference citations when needed.

Let's try to get these applications on file during September. If you believe Lesley or Beth should review the draft as well, please make them a copy.

Thanks for your time.

Cheers, LYNN

SELECTABLE MARKER GENES

Inventors

Susanne Pippig

Gabor Veres

BACKGROUND OF THE INVENTION

This invention relates to a method of using a muscle specific tyrosine kinase receptor molecule (MuSK-R) or a mutated MuSK-R thereof as a selectable cell marker for diverse cell types and particularly in cells of hematopoietic origin.

The use of selectable markers is well known for the identification of prokaryotic and eukaryotic cells, and the use of these markers is essential because frequently when a DNA sequence of interest is introduced into a cell it will not necessarily lead to a phenotype that is readily determined. The number of selectable markers used in identifying eukaryotic cells and especially mammalian cells has been limited. In the past, selectable markers that conferred drug resistance have been employed (i.e. G-418 and hygromycin). More recently, selectable markers that are combined with fluorescence activated cell sorting (FACS) have been used, for example, green fluorescent protein (GFP). Alternatively, antibodies that recognize a cell surface molecule may be coupled to a fluorophore to help identify the cells of interest.

Several cell surface molecules have been used as selectable cell markers including murine CD8, CD24, and human Low-Affinity Nerve Growth Factor Receptor (NGFR). Reference is made to the following publications; WO95/06723; WO98/19540; Jolly et al., (1983) Proc. Natl. Acad. Aca. 80: 477; Reddy et al., Mol. Brain Res. (1990) 8:137, and Valenzuela et al., (1995) Neuron 15: 573. Some of these cell surface molecules have been

mutated in their intracellular domain to avoid signaling of the molecules when binding to their ligand. (However upon ligand binding some of the intracellularly mutated molecules may homo-, heterodimerize or trimerize. If a newly introduced molecule in the cell should heterodimerize with endogenous receptors a dominant negative effect may result.

not clear if Musk can hetero multimerize

physiology of the cell could be disturbed

5 (WHY IS THIS SIGNIFICANT?) The present invention provides an improvement (OR IS DIFFERENT FROM THE USE OF NGFR AS A SELECTABLE MARKER

BECAUSE --- please fill in by using Musk in a non-muscle cell, ligand binding will be prevented

=> molecule is inactive

10

SUMMARY OF THE INVENTION

Accordingly, the invention provides for a method of using a muscle specific tyrosine kinase receptor molecule (MuSK-R) or a mutant receptor molecule thereof as a selectable marker in a mammalian cell. This method comprises incorporating a nucleic acid sequence encoding the coding region of a MuSK-R or a mutated MuSK-R into a population of mammalian cells; allowing expression of the nucleic acid sequence in the mammalian cells, such that the cells are not capable of signal transduction, and selecting the cells expressing MuSK-R or mutated MuSK-R. In one embodiment the mutated MuSK-R molecule is a MuSK-R sequence having at least 150 amino acids deleted from the intracellular domain. In another embodiment the mutated MuSK-R molecule is a MuSK-R sequence having at least 300 amino acids deleted from the intracellular domain. In yet another embodiment the mutated MuSK-R molecule is a MuSK-R sequence having the kinase catalytic site deleted. Preferred mutant MuSK-R molecules are disclosed as MuSK-RI and MuSK-RII. In one embodiment the MuSK-R or mutant MuSK-R is introduced on a retroviral vector. In another embodiment the nucleic acid encoding the MuSK-R or mutant MuSK-R is introduced in combination with a second nucleic acid encoding sequence wherein the second sequence encodes a protein of interest.

30 In another aspect, the invention provides a method of selecting genetically modified mammalian cells. This method comprises incorporating a nucleic acid encoding a muscle specific tyrosine kinase receptor (MuSK-R) or mutated MuSK-R into a

population of mammalian cells; introducing a heterologous DNA sequence which encodes a protein of interest into the population of mammalian cells; allowing expression of the MuSK-R or mutated MuSK-R in said cells; and selecting genetically modified mammalian cells expressing said MuSK-R or mutated MuSK-R. In one embodiment the
5 heterologous DNA sequence encoding the protein of interest is introduced on the same vector as the MuSK-R or mutated MuSK-R.

In yet another aspect, the invention includes a method for the immunoselection of transduced mammalian cells comprising, transducing cells with a nucleic acid sequence
10 encoding a muscle specific tyrosine kinase molecule (MuSK-R) or a mutated MuSK-R molecule; incubating the cells with a marked antibody which recognizes and binds specifically to the MuSK-R or mutated MuSK-R; and identifying the marked transduced cells. In one embodiment the method further comprises separating the identified transduced cells. In yet a further embodiment the method further comprises expanding the
15 marked transduced cells.

A further aspect of the invention includes a method of identifying mammalian cells expressing a protein of interest, comprising introducing into a population of mammalian cells a nucleic acid encoding a muscle specific tyrosine kinase molecule
20 (MuSK-R) or a mutated MuSK-R molecule, wherein said MuSK-R or mutant MuSK-R can not effect signal transduction; introducing a nucleic acid comprising a DNA sequence encoding a protein of interest into said population; culturing the mammalian cells under conditions which favor growth and expansion of said cells; and selecting cells which express MuSK-R or mutated MuSK-R thereby obtaining cells which have the capable of
25 expressing a protein of interest.

BRIEF DESCRIPTION OF THE DRAWINGS

30 Figure 1 is a schematic representation of a wild-type (WT) MuSK-R molecule, corresponding to SEQ ID NO. 1 and two mutated MuSK-R molecules. The mutated

MuSK-R molecules are MuSK-RI having amino acid residues 537 - 879 deleted from the WT-MuSK-R and MuSK-RII having amino acid residues 557 - 879 deleted from the WT-MuSK-R.

10 aa insert 210 - 219 | 9 aa insert in XC domain 464 - 472

5 Figure 2 illustrates the nucleotide sequence and deduced amino acid sequence of human WT MuSK-R and corresponds to SEQ ID NO: 1. The extracellular domain includes amino acid residues 1 - ^{19 signal peptide} ~~120~~ ⁵⁰³, the transmembrane domain includes amino acid residues ⁵⁰⁴ to ⁵²⁵, and the cytoplasmic domain includes amino acid residues ⁵²⁶ to ⁸⁷⁹. **PLEASE PROVIDE THE SEQUENCE. ARE THERE OTHER**

10 **FEATURES THAT HAVE SIGNIFICANCE THAT WE SHOULD POINT OUT??**

This includes 10/9 aa insert.

Figure 3 illustrates expression of the WT MuSK-R (SEQ ID NO: 1) MuSK-RI and MuSK-RII on CEN33 cells after transduction with supernatants that were generated from PPA-6 cells. **(PLEASE PROVIDE THIS FIGURE OR SIMILAR**

15 **INFORMATION))**

MuSK that we are using does not contain 9 aa insert

DETAILED DESCRIPTION OF THE INVENTION

20 **A. THE SEQUENCES**

The selective marker of the instant invention is a muscle specific tyrosine kinase receptor (MuSK-R) molecule or a mutation thereof. MuSK-R molecules are members of the tyrosine kinase receptor family. In general, the extracellular domain of these
25 molecules provide a ligand-binding site. It is believed the binding of the ligand results in signal transduction involving the intracellular catalytic domain and a signal is then transmitted to target proteins. Most protein kinase receptors share a conserved intracellular catalytic domain. The extracellular ligand-binding domain is the most distinctive feature ^{after the tyrosine kinase domain} of the protein kinase receptors and various classifications of the
30 tyrosine kinase molecules are based on this structure. (See van der Geer et al., (1994) Annu. Rev. Cell Biol. 10:251).

The domain structure of a wild-type MuSK is schematically illustrated in Figure 1. MuSK is comprised of a signal sequence that targets the protein to the secretory pathway. (IS THE SIGNAL SEQUENCE CONSIDERED PART OF THE
5 EXTRACELLULAR DOMAIN?) The extracellular domain follows the signal sequence. ^{~ 500 aa} This domain is made up of several hundred amino acids (HOW MANY - RANGE). The extracellular domain means the part of the receptor that normally projects from the cell into the extracellular environment and includes the ligand binding region. In MuSK the extracellular domain contains immunoglobulin-like (Ig-like) regions. ~~Naturally~~ Four Ig-
10 like regions are found. The extracellular domain also may include a C6-box (IS THIS CORRECT?? ARE THERE 4 IG REGIONS? WHAT IS A C6-BOX?? WHAT OTHER DISCTINCTIVE FEATURES ARE THERE? A KRINGLE MOTIF?? WHAT ABOUT CYS RICH REGIONS??). The transmembrane domain is generally localized in the cell membrane and consists of a stretch of hydrophobic residues followed by several basic
15 residues. The intracellular domain (also referred to as the cytoplasmic domain) includes the catalytic part of the molecule and is positioned within the cell. (IS THE ID THE SAME AS THE CATALYTIC D OR IS THE CATALYTIC D CONSIDERED A SUBSET OF THE ID?) WHAT ARE SIGNIFICANT PROPERTIES OF TYROSINE KINASE RECEPTOR MOLECULES THAT WOULD FALL WITHIN THIS GROUP
20 OF RECEPTORS?) I don't understand this part

MuSk-Rs are also known in the art as denervated muscle kinase receptors (DmK-Rs) and reference is made to U.S. Pat. No. 5,656,473. MuSK-R sequences have been
25 isolated and identified from humans, rats, mice, ^{xenopus *} (OTHERS??). Reference is also made to EMBL/GenBank Accession Numbers:
U34985. Closely related to human MuSK-R is a receptor isolated from the electric ray *Torpedo californica* designated Torpedo tyrosine kinase receptor. (Jennings, et al. Proc. Natl. Acad. Sci. USA 90:2895 (1993)). MuSK-R is
30 specific to the skeletal muscle lineage. It is expressed early in the muscle lineage and becomes localized to the motor endplate as muscle matures.

* Fu AK, Smith JD, Zhou H, Chu FH, Tsim KW, Peng BH, Ip NY

CONFIDENTIAL/PRIVELEGED DOCUMENT- DO NOT COPY

xenopus muscle-specific kinase: molecular cloning and prominent expression in neural tissues during early embryonic development

no?
373-382
= C6 box
Feb, 11(2):
J. Neurosci 1999

As used herein "MuSK-R and mutated MuSK-R" refer to nucleotides or protein as appropriate from context. Polynucleotides of the invention may be in the form of RNA or in the form of DNA, which DNA includes cDNA, genomic DNA or synthetic DNA.

5

The MuSK-Rs used in the present invention include those molecules known as DmK receptors and include the family of tyrosine kinase receptors closely related to MuSK-R wherein the extracellular domain has similar structural properties. These closely related receptors are, for example, Torpedo tyrosine kinase receptors and ROR tyrosine kinase receptors (WHAT DOES THE ABBREVIATION ROR MEAN??? PLEASE PROVIDE A REFERENCE * WHAT ARE THE COMMON FEATURES? THESE MUST BE DESCRIBED TO GET COVERAGE - ALSO CAN YOU POSTULATE ON THE DEGREE OF HOMOLOGY OF THE EXTRACELLULAR DOMAINS) ROR 3 Ig-like domains, CG-spacer position of these domains are similar to MuSK + Torpedo RTK

15

The MuSK-Rs or mutant MuSK-Rs thereof which are used as selective markers in the present invention are molecules wherein the receptor no longer possesses signaling activity. PLEASE DEFINE SIGNAL ACTIVITY/ SIGNAL TRANSDUCTION triggering a response pathway in the cytosol and to the nucleus which ultimately leads to activation of transcription

20

The lack of signaling activity may be due to a) use of a wild-type MuSK-R in tissue or cells other than muscle or b) use of a mutated MuSK-R. While modifications of MuSK-Rs are known, the use of MuSK-Rs or mutated MuSK-Rs thereof as selectable marker is not known.

25

It is well known that the localization of MuSK-R is in muscle tissue and further that MuSK serves as the functional agrin receptor. Agrin is a nerve-derived factor that can induce molecular reorganizations at the motor endplate. (See Glass, et al. Cell 85:513 (1996)), therefore use of a wild-type MuSK-R may be used as a selective marker in tissue other than muscle.

30

not correct (Wild-type MuSK-Rs include not only naturally occurring MuSK-Rs but also may include genetically engineered MuSK-Rs.) Additionally, wild-type MuSK-Rs may include receptor molecules wherein the wild-type MuSK-R has undergone changes in the DNA sequence that do not significantly effect the properties of the protein. These changes include ones that do not change the encoded amino acid sequence, ones that result in conservative substitutions of amino acid sequences, or ones that result in one or a few amino acid deletions or additions. Suitable substitutions are known by those skilled in the art. Amino acid residues that can be conservatively substituted for one another include, but are not limited to, glycine/alanine; valine/isoleucine/leucine, asparagine/glutamine; aspartic acid/glutamic acid; serine/threonine; lysine/arginine; and phenylalanine/tyrosine. Any conservative amino acid substitution not significantly affecting the properties of a wild-type MuSK-R is encompassed by the present invention.

15 Mutated MuSK-Rs used in the invention include truncations and/or deletions of wild-type MuSK-Rs. The mutation may occur in the extracellular domain and/or the intracellular domain by means well known in the art. The mutation causes the molecule to be devoid of signaling activity. However, the extracellular domain is still capable of binding an antibody.

20

In one nonlimiting embodiment, the wild-type MuSK-R molecule is the sequence having Accession Number U34985 and corresponds to Figure 2. (IS THIS ENBL OR GENBANK??? IS THIS THE SAME SEQUENCE FOUND IN THE CLONE U34985 DEPOSITED WITH ATCC # 75498 ?? The extracellular domain is encoded by nucleotides 1 through 1509, the transmembrane domain is encoded by nucleotides 1510 through 1575, and the intracellular domain is encoded by nucleotides 1576 through 2637 PLEASE FILL IN THE BLANKS)

1 * discloses deletion mutants of MuSK-R. (PLEASE 2 truncated Musk each 300 aa of 1c domain
30 PROVIDE A REFERENCE) One mutation (referred to as *2) -Others

Neuron, Vol. 18, 623-635, April 1997: Rapsyn is required for Musk signaling and recruits synaptic components to a Musk-containing scaffold

CONFIDENTIAL/PRIVILEGED DOCUMENT- DO NOT COPY
Elizabeth D. Apel, David J. Glass, Lisa M. Moscoso, George D. Yancopoulos,
and Joshua R. Sanes

Preferred truncations of wild-type MuSK-Rs used in the methods of the instant invention include deletion of at least 150, preferably at least 200, more preferably at least 250, and most preferably at least 300 amino acids of the cytoplasmic domain. The truncations may include deletion of tyrosine phosphorylation sites in the range of 2 to 7 sites and deletion of the kinase catalytic site. (IS THIS TRUE??)

In this respect, a particularly preferred Mutated MuSK-R is designated MuSK-RI wherein amino acid sequence 537 to 879 of the cytoplasmic domain illustrated in Figure 2 is deleted. Another particularly preferred Mutated MuSK-R is designated MuSK-RII wherein amino acid sequence 557 to 879 of the cytoplasmic domain illustrated in Figure 2 is deleted. *525 - 879 I just get rid of kinase insert*

CAN WE LIST OTHER TYPES OF TRUNCATIONS THAT COULD WORK EVEN IF NOT MADE??? IS IT IMPORTANT TO MAINTAIN A PART OF THE CYTOPLASMIC DOMAIN INTACT ?? AT THE TRANSMEMBRANE JUNCTION OR AT THE C-END?? *just so that the protein can be stably expressed*

In addition to modification of the cytoplasmic domain mutations may be made in the extracellular domain. The extracellular domain modification may include deletion of at least about 100 amino acids, preferably at least about 150 amino acids, more preferably at least about 200 amino acids, and still more preferably at least about 250 amino acids. In one embodiment the modification will include removal of (LET'S TALK ABOUT THIS!)

The wild-type MuSK-Rs and the mutated MuSK-Rs must contain an antibody binding site in the extracellular domain. (CAN YOU ELABORATE ON THIS - HOW LARGE MUST THE SITE BE? ETC)

30

B. DETAILS ON METHODS OF MUTATING SEQUENCES

ISBN

MuSK-R molecules including molecules designated as DmK-R, Torpedo tyrosine kinase receptors, ROR tyrosine kinase receptors and mutants thereof may be obtained from well-known sources such as various sequence databases including GenBank

General strategies for creating mutations in genes are well known and these methods may be used to create Mutant MuSK-Rs useful in the present invention. Both random and site-directed methods may be effective to create mutations in wild type MuSK-Rs. Random methods encompass altering the sequences within restriction endonuclease sites, inserting an oligonucleotide linker randomly into a plasmid, using chemicals to damage plasmid DNA, and incorporating incorrect nucleotides during in vitro DNA synthesis. However, directed mutagenesis may be a more beneficial tool. These methods are also well known in the art and include oligonucleotide directed mutagenesis by using degenerate oligonucleotides for random mutations at a specific site and mutagenesis by gene synthesis. References which detail these methods are well known and include, Wu et al., eds. Methods in Enzymology, Vol. 154: Recombinant DNA, Part E, Academic, NY 1987 (IS THIS ENOUGH DETAIL? SINCE THE INVENTION CONCERNS USER OF MUTATED SEQUENCES, I BELIEVE WE SHOULD PROVIDE MORE DETAIL ON HOW ONE SKILLED IN THE ART WOULD MUTATE MuSK-R SEQUENCES. IF THIS IS REALLY STANDARD PROCEDURE, LETS SUPPLY A FEW MORE REFERENCES)

The usefulness of a MuSK-R or mutant thereof as a selectable marker concerns the ability to select genetically modified cells both in vitro and in vivo. While the MuSK-R or mutant thereof may be introduced into a target cell as part of a nucleic acid construct operatively linked to a promoter sequence, in a preferred embodiment the MuSK-R or mutant thereof is placed in a vector and then introduced to a target cell.

C. DETAILS ON CLONING THE MUTATED SEQUENCES INTO VECTORS

Vectors containing both a promoter and a cloning site into which a polynucleotide can be operatively linked are well known in the art. Such vectors are capable of transcribing RNA in vitro or in vivo, and are commercially available from sources such as Stratagene (La Jolla, CA) and Promega Biotech (Madison, WI). Examples of vectors include vectors derived from viruses, such as baculovirus, retroviruses, adenoviruses, adeno-associated viruses, and herpes simplex viruses; bacteriophages; cosmids; plasmid vectors; fungal vectors; synthetic vectors; and other recombination vehicles typically used in the art. These vectors have been described for expression in a variety of eukaryotic and prokaryotic hosts and may be used for simple protein expression.

In a preferred embodiment, the vector comprises a polynucleotide operatively linked to a regulatory sequence. Regulatory sequences include promoters, enhancers, polyadenylation signals, and other expression control elements. The promoter may be either a prokaryotic or eukaryotic promoter. The vector may further comprise a polyadenylation signal that is position 3' of the carboxy-terminal amino acid. Vectors containing both a promoter and a cloning site into which a polynucleotide can be operably linked are well known in the art. Such vectors are capable of transcribing RNA in vitro or in vivo, and are commercially available from sources such as Stratagene (La Jolla, CA) and Promega Biotech (Madison, WI). Specific examples include pSG, pSV2CAT, and pXt1 from Stratagene and pMSG, pSVL, pBPV and pSVK3 from Pharmacia. Other exemplary vectors include the pCMV mammalian expression vectors, such as pCMV6b and pCMV6c (Chiron Corporation), pSFFV-Neo, and pBluescript-SK+. In order to optimize expression and/or in vitro transcription, it may be necessary to remove, add or alter 5' and/or 3' untranslated portions of polynucleotides to eliminate potentially extra inappropriate alternative translation initiation codons or other sequences that may interfere with or reduce expression, either at the level of transcription or translation. Alternatively consensus ribosome binding sites can be inserted immediately 5' of the start codon to enhance expression.

30

Particularly preferred vectors are retroviral vectors and reference is made to Coffin et al., "Retroviruses", (1997) Chapter 9 pp; 437-473 Cold Springs Harbor Laboratory Press. Retroviral vectors useful in the invention are produced recombinantly by procedures already taught in the art. WO94/29438, WO97/21824 and WO97/21825 describe the construction of retroviral packaging plasmids and packing cell lines. Common retroviral vectors are those derived from murine, avian or primate retroviruses. The most common retroviral vectors are those based on the Moloney murine leukemia virus (MoMLV) and mouse stem cell virus (MSCV). Vectors derived from MoMLV include, Lmily, LINGFER, MINGFR and MINT (Chang et al., 1998, Blood 92:1 - 11). Further non-limiting examples of vectors include those based on Gibbon ape leukemia virus (GALV), Moloney murine sarcoma virus (MoMSV), and the lentiviruses, such as Human immunodeficiency virus (HIV-1 and HIV-2). Vectors derived from MSCV include MSCV-MiLy (Agarwal et al., J. of Virology 72:3720). New vector systems are continually being developed to take advantage of particular properties of parent retroviruses such as host range, usage of alternative cell surface receptors and the like. The present invention is not limited to particular retroviral vectors, but may include any retroviral vector. Particularly preferred vectors include DNA from a murine virus corresponding to two long terminal repeats, and a packaging signal. In one embodiment the vector is a MoMLV or MSCV derived vector.

only
envelope
is used

20

In producing retroviral vector constructs, the viral gag, pol and env sequence will generally be removed from the virus, creating room for insertion of foreign DNA sequences. Genes encoded by foreign DNA are usually expressed under the control a strong viral promoter in the long terminal repeat (LTR). Numerous promoters are known in addition to the promoter of the LTR. Non-limiting examples include the phage lamda PL promoter, the human cytomegalovirus (CMV) immediate early promoter; the U3 region promoter of the Moloney Murine Sarcoma Virus (MMSV), Rous Sarcoma Virus (RSV), or Spleen Focus Forming Virus (SFFV); Granzyme A promoter; CD34 promoter; and the CD8 promoter. Additionally inducible or multiple promoters may be used. In a preferred embodiment the promoter for the vector which comprises the mutated PTKR

30

will be selected from the following group _____

(THIS IS VERY IMPORTANT. PLEASE LIST THE PROMOTERS WHICH
CAN BE USED FOR:

5 A) THE MUTATED MuSK-R SEQUENCE - INCLUDING THE ACTUAL
 PROMOTER USED -

B) THE PROTEIN OF INTEREST and

C) THE RETROVIRAL VECTOR - LTR.

10 PLEASE ALSO PROVIDE REFERENCES. IT IS IMPORTANT TO BE
 COMPREHENSIVE IN THIS LIST - AS IN THE FIRST INSTANCE THE
 EXAMINER MAY WANT TO LIMIT THE PROMOTERS TO THOSE
 LISTED

15 Such a construct can be packed into viral particles efficiently if the gag, pol and
env functions are provided in trans by a packaging cell line. Therefore when the vector
construct is introduced into the packaging cell, the gag-pol and env proteins produced by
the cell, assemble with the vector RNA to produce infectious virions that are secreted into
the culture medium. The virus thus produced can infect and integrate into the DNA of the
20 target cell, but does not produce infectious viral particles since it is lacking essential
packaging sequences. Most of the packing cell lines currently in use have been
transfected with separate plasmids, each containing one of the necessary coding
sequences, so that multiple recombination events are necessary before a replication
competent virus can be produced. Alternatively the packaging cell line harbors a
25 provirus. (The DNA form of the reverse-transcribed RNA once its integrates into the
genomic DNA of the infected cell). The provirus has been crippled so that although it
may produce all the proteins required to assemble infectious viruses, its own RNA can
not be packaged into virus. RNA produced from the recombinant virus is packaged
instead. Therefore, the virus stock released from the packaging cells contains only
30 recombinant virus. Non-limiting examples of retroviral packaging lines include PA12,
PA317, PE501, PG13, ΨCRIP, RD114, GP7C-tTA-G10, ProPak-A (PPA-6), and PT67.

Reference is made to Miller et al., *Mol. Cell Biol.* 6:2895 (1986); Miller, et al., *Biotechniques* 7:980 (1989); Danos et al., *Proc. Natl. Acad. Sci. USA* 85:6460 (1988); Pear et al., *Proc. Natl. Acad. Sci. USA* 90:8392 (1993); Rigg et al., *Virology* 218:290 (1996); and Finer et al., *Blood* 83:43 (1994).

5

Additionally preferred vectors include adenoviral vectors (See Frey et al., *Blood* 91:2781 (1998) and WO95/27071) and adeno-associated viral vectors (See Chatterjee et al., *Current Topics in Microbiol. and Immunol.* 218:61 (1996). Reference is also made to Shenk, Chapter 6, 161 – 178, Breakefield et al., Chapter 8 201-235; Kroner-Lux et al., Chapter 9, 235 – 256 in *Stem Cell Biology and Gene Therapy*, eds. Quesenberry et al., John Wiley & Sons, 1998 and U.S. Pat. Nos. 5,693,531 and 5,691,176. The use of adenovirus derived vectors may be advantageous under certain situations because they are not capable of infecting non-dividing cells, and unlike retroviral DNA, the adenoviral DNA is not integrated into the genome of the target cell. Further the capacity to carry foreign DNA is much larger in adenoviral vectors than retroviral vectors. The adeno-associated viral vectors are another useful delivery system. The DNA of these viruses may be integrated into non-dividing cells, and a number of polynucleotides have been successfully introduced into different cell types using adeno-associated viral vectors.

20 In one embodiment, the construct or vector will include not only a nucleic acid sequence encoding a MuSK-R or mutant thereof as a selective marker but also a second nucleic acid sequence encoding a protein of interest to be transferred into a target cell. In a preferred embodiment the nucleic acid molecules are DNA.

25 The protein of interest is broadly defined and includes for example, a therapeutic protein, a structural gene, a ribozyme, or an antisense sequence. The structural protein or gene may be the entire protein or only the functionally active fragment thereof. The protein may include for example one that regulates cell differentiation or a therapeutic gene capable of compensating for a deficiency in a patient that arises from a defective endogenous gene. Gene means a nucleic acid molecule the sequence which includes all the information required for the normal regulated production of a particular protein

30

including the structural coding sequence. Additionally a therapeutic protein or gene may be one that antagonizes production or function of an infectious agent, antagonizes pathological processes, improves a host's genetic makeup, or facilitates engraftment.

5 Specific examples of a therapeutic gene or gene sequences are ones effective in the treatment of adenosine deaminase deficiency (ADA); sickle cell anemia; recombinase deficiency; recombinase regulatory gene deficiency; HIV such as an antisense or trans-dominant REV gene or a gene carrying a herpes simplex virus thymidine kinase (HSV-tk)). The second DNA sequence may encode new antigens or drug resistant genes or may
10 encode a toxin or an apoptosis inducer effective to specifically kill cancerous cells, or a specific suicide gene to hematopoietic cells may be included.

 The vector or construct may also comprise, besides the second nucleic acid sequence encoding the protein of interest, a further DNA sequence. More than one gene
15 may be necessary for the treatment of a particular disease. Alternatively more than one gene can be delivered using several compatible vectors. Depending on the genetic defect, the therapeutic gene can include regulatory and untranslated sequences. For human patients the therapeutic gene will generally be of human origin although genes of closely related species that exhibit high homology and biologically identical or equivalent
20 function in humans may be used if the gene does not produce an adverse immune reaction in the recipient.

 Nucleotide sequences for the protein of interest or further DNA sequence will generally be known in the art or can be obtained from various sequence databases such as
25 GenBank. One skilled in the art will readily recognize that any structural gene can be excised as a compatible restriction fragment and placed in a vector in such a manner as to allow proper expression of the structural gene in hematopoietic cells.

D. TARGET CELLS

30

The target cells of the invention are mammalian cells and these include but are not limited to humans, mice, monkeys, farm animals, sport animals, pets, and other laboratory rodents and animals. Preferably the target cells are human cells. Preferred human cells include liver, hematopoietic, and (WHAT ARE OTHER CELL TYPES

5 WHICH CAN BE USED??? PLEASE LIST) cells. Hematopoietic cells encompass hematopoietic stem cells, erythrocytes, neutrophils, monocytes, platelets, mast cells, eosinophils and basophils, B and T lymphocytes and NK cells as well as the respective lineage progenitor cells. Hematopoietic stem cells and T-cells are especially preferred. Hematopoietic stem cells are defined as a population of hematopoietic cells
10 containing long term multilineage repopulating potential. T-cells are defined as a type of lymphocyte and are thought to develop from hematopoietic stem cells. There are many types of T-cells including cytotoxic T-cells, helper T-cells, inducer T-cells and supressor T cells.

15 Methods of obtaining hematopoietic cells are well known in the art and not repeated herein. Non-limiting sources of hematopoietic cells, including hematopoietic stem cells, are bone marrow, embryonic yolk sac, fetal liver tissue, adult spleen, and blood such as adult peripheral blood and umbilical cord blood. (To et al., Blood 89:2233 (1997)). Bone marrow cells may be obtained from ilium, sternum, tibiae, femora, spine
20 and other bone cavities.

The manner in which hematopoietic cells may be separated from other cells is not critical to this invention. Various procedures may be employed and include physical separation, magnetic separation using antibody-coated magnetic beads, affinity
25 chromatography, and cytotoxic agents joined to a monoclonal antibody or used in conjunction with a monoclonal antibody. Also included is the use of fluorescence activated cell sorters (FACS) wherein the cells can be separated on the basis of the level of staining of the particular antigens. These techniques are well known to those skilled in the art and are described in various references including U.S. Patent Nos. 5,061,620;
30 5,409,8213; 5,677,136; and 5,750,397; and Yau et al. (1990) Exp. Hematol. 18:219-222.

The order of cell separation is not critical to the invention, and specific cell types may be separated either prior to genetic modification with the mutated cell surface receptor or after genetic modification. Preferably cells are initially separated by a coarse separation followed by using positive and/or negative selection. In humans the surface antigen expression profile of an enriched hematopoietic stem cell population may be identified by CD34⁺Thy-1⁺Lin⁻. Other nonlimiting enriched phenotypes may include: CD2⁻, CD3⁻, CD4⁻, CD8⁻, CD10⁻, CD14⁻, CD15⁻, CD19⁻, CD20⁻, CD33⁻, CD34⁻, CD38^{lo/-}, CD45RA⁻, CD59^{+/-}, CD71⁻, CDW109⁺, glycophorin⁻, AC133⁺, HLA-DR^{+/-}, and EM⁺. Lin⁻ refers to a cell population selected on the basis of lack of expression of at least one lineage specific marker, such as, CD2, CD3, CD14, CD15 and CD56. The combination of expression markers used to isolate and define an enriched HSC population may vary depending on various factors and may vary as other express markers become available.

Murine HSCs with similar properties to the human CD34⁺Thy-1⁺Lin⁻ may be identified by kit⁺Thy-1.1^{lo}Lin^{-/lo}Sca-1⁺ (KTLS). Other phenotypes are well known. When CD34 expression is combined with selection for Thy-1, a composition comprising approximately fewer than 5% lineage committed cells can be isolated (U.S. Patent No. 5,061,620).

It has been shown that CD34 is expressed on most T cells, and that these cells lack cell surface expression of CD1, CD2, CD3, CD4, and CD8 antigens. Also CD54RA is a useful T cell marker. The most well known T cell marker is the T cell antigen receptor (TCR). There are presently two defined types of TCRs. TCR-2 (consisting of α and β polypeptides) and TCR-1 (consisting of δ and γ polypeptides). B cells may be selected, for example, by expression of CD19 and CD20. Myeloid cells may be selected for example, by expression of CD14, CD15 and CD16. NK cells may be selected based on expression of CD56 and CD16. Erythrocytes may be identified by expression of glycophorin A. One skilled in the art is aware of other useful markers for identification of various cell types. (DO YOU HAVE EXAMPLES FOR OTHER PREFERRED TARGET CELLS BESIDES HC i.e. KIDNEY CELLS, LIVER CELLS etc.)

Once a population containing the target cells are harvested and target cells particularly hematopoietic stem cells or T-cells separated, the cells are cultured in a suitable medium comprising a combination of growth factors that are sufficient to maintain growth. Methods for culturing hematopoietic cells are well known to those skilled in the art, and these methods are only briefly mentioned herein. (IS THIS TRUE FOR OTHER CELL TYPES? DO YOU HAVE A REFERENCE?) Any suitable culture container may be used, and these are readily available from commercial vendors. The seeding level is not critical and will depend on the type of cells used, but in general the seeding level will be at least 10 cells per ml, more usually at least about 100 cells per ml and generally not more than 10^6 cells per ml when the cells express CD34.

Various culture media can be used and non-limiting examples include IMDM, X-vivo 15 and RPMI-1640. These are commercially available from various vendors. The formulations may be supplemented with a variety of different nutrients, growth factors, such as cytokines and the like. The medium can be serum free or supplemented with suitable amounts of serum such as fetal calf serum, autologous serum or plasma. If cells or cellular products are to be used in humans, the medium will preferably be serum free or supplemented with autologous serum or plasma. (Lansdorp et al., (1992) J. Exp. Med. 175:1501 and Petzer et al. (1996) PNAS 93:1470).

Non-limiting examples of compounds which may be used to supplement the culture medium are TPO, FL, KL, IL-1, IL-2, IL-3, IL-6, IL-12, IL-11, stem cell factor, G-CSF, GM-CSF, Stl, MCGF, LIF MIP-1 α and EPO. These compounds may be used alone or in any combination, and preferred concentration ranges may be readily determined from the published art. When murine stem cells are cultured, a preferred non-limiting medium includes mIL-3, mIL-6 and mSCF.

Other molecules can be added to the culture media, for instance, adhesion molecules, such as fibronectin or RetroNectin™ (commercially produced by Takara Shuzo Co., Otsu Shigi, Japan).

5 In vitro systems for measurement of mammalian stem cell activity include the long-term culture initiating cell assay (LTCIC) and the cobblestone-area-forming cell (CAFC) assay. (Pettengell et al., Blood 84:3653 (1994); Breems et al., Leukemia 8:1095 (1994); Reading, et al., Exp. Hem. 22:786 (Abst # 406) (1994); and Ploemacher et al., Blood 74:2755 (1989)). In the CAFC assay a sparsely plated cell population is simply
10 tested for its ability to form distinct clonal outgrowths (or cobblestone areas) on a stromal cell monolayer over a period of time. This assay gives frequency readouts that correlate with LTCIC and are predictive of engraftment in in vivo assays and patients. A particularly preferred CAFC assay is described in Young et al., Blood 88:1619 (1996). Flow cytometry can be used to subset hematopoietic cells from various tissue sources by
15 the surface antigens they express. A combination of these assays may be used to test for hematopoietic cells or stem cells.

 In one preferred embodiment the invention concerns a method of using a polynucleotide sequence encoding a MuSK-R or mutant thereof as a selectable marker. In
20 a most preferred embodiment the polynucleotide sequence encodes MuSK-RI, MuSK-RII or a polynucleotide sequence similar thereto with minor changes. A polynucleotide is said to "encode" a polypeptide if, in its native state or when manipulated by methods well known to those of skill in the art it can be transcribed and/or translated to reproduce a polypeptide or fragment thereof. A construct or vector including the MuSK-R or mutant
25 thereof may be incorporated into the target population by any means of genetic transfer or modification known in the art.

E. GENETIC TRANSFER OF THE MUTATED SEQUENCE INTO THE
TARGET CELLS) TRANSDUCTION, LIPOSOME TRANSFER ETC)

30

The term "genetic modification" refers to any addition, deletion or disruption to a cells normal nucleotides and the methods of genetic modification are intended to encompass any genetic modification method of exogenous or foreign gene transfer or nucleic acid transfer into mammalian cells (particularly human hematopoietic cells). The
5 term includes but is not limited to transduction (viral mediated transfer of host DNA from a host or donor to a recipient, either in vivo or ex vivo), transfection (transformation of cells with isolated viral DNA genomes), liposome mediated transfer, electroportation, calcium phosphate coprecipitation and others. Methods of transduction include direct co-culture of cells with producer cells (Bregni et al. (1992), Blood 80:1418 – 1422) or
10 culturing with viral supernatant alone with or without appropriate growth factors and polycations (Xu et al., 1994, Exp. Hemat. 22:223 – 230).

In a preferred embodiment the target cells are transduced with a retroviral vector as previously described. The host cell range that may be infected is determined by the
15 viral envelope protein. The recombinant virus can be used to infect virtually any other cell type recognized by the env protein provided by the packaging cell, resulting in the integration of the viral genome in the transduced cell and the stable incorporation of the foreign gene product. In general, murine ecotropic env of MoMLV allows infection of rodents cells, whereas amphotropic env allows infection of rodent, avian and some
20 primate cells including human cells. Amphotropic packaging of cell lines for use with MoMLV systems are known in the art and are commercially available. These include but are not limited to, PA12, PA317, ψ CRIP, and FLYA13. (See, Miller et al. (1985) Mol. Cell Biol. 5:431 – 437; Mill et al. (1986) Mol. Cell Biol. 6:2895 – 2902; and Danos et al. (1988) Proc.Natl. Acad. Sci. USA 85:6460 – 6464. Recently, the G-glycoprotein from
25 vesicular stomatitis virus (VSV-G) has been substituted for the MoMLV env protein. (See Burns et al. (1993) Proc.Natl. Acad. Sci. USA 90:8033-8037; and WO92/14829). Xenotropic vector systems also exist which allow infection of human cells.

30 F. SELECTION PROCESSES

THIS SECTION NEEDS WORK.....

ONCE THE CELLS ARE TRANSFORMED (HOW ARE THE CELLS WHICH INCLUDE THE MuSK-R MARKER IDENTIFIED??)

5 I ASSUME WE USE ANTIBODIES -BUT ARE THERE OTHER WAYS TO IDENTIFY THE CELLS - THESE MUST BE LISTED OTHERWISE THE EXAMINER MAY LIMIT THE CLAIMS TO THE USE OF ANTIBODIES ONLY

1. IMMUNOSELECTION

2.

10 3.

4.

The genetically modified cells of the present invention may be easily separated by the use of antibodies. Antibodies may be obtained by methods well known in the art by immunizing an animal with a protein encoding DNA according to the invention and isolating the antibodies from the serum of the immunized animal. (reference is made to Cite a book/article etc

(THIS NEEDS EXPANSION - I ASSUME ANY ANTIBODY THAT BINDS WITH A EGFR MOLECULE WOULD WORK BECAUSE THIS PART OF THE EXTRACELLULAR DOMAIN MUST REMAIN INTACT!)

The antibody may be a monoclonal antibody or a polyclonal antibody. Monoclonal antibodies to MuSK-R are available commercially (FROM WHERE)

25 The antibody may be identified and assayed in vitro by a range of methods including gel diffusion, immunoassay, immunoelectrophoresis and immunofluorescence.

Once the modified cells are labeled they can be incubated with an antibody against the mutated receptor. The labeled cells are then separated from the unbound cells.

30 (HOW - GENERAL DESCRIPTION OR REFERENCE)

Southern analysis

Northern blotting

5 FACS analysis

G. GENERAL CONCLUSION TYPE THINGS

The target cells containing a MuSK-R or mutant thereof and optionally a second
10 DNA sequence encoding a protein of interest may be expanded either prior to or after
selection by means well known in the art as generally described in _____;
_____ and _____. **(OR DESCRIBE THE WAYS)**

The marked cells may further be used in an autologous or allogeneic setting
15 wherein the hematopoietic cells, preferably stem cells or T-cells are expanded and then
used for example in bone marrow transplantation, graft facilitation, or immune
reconstitution. The expanded cells including the mutated cell surface marker may be
refused into a subject ***I Samples may be taken and then tested for
the selective marker by _____ to determine and further assess efficiency***
20 of transduction.

The practice of the present invention will employ, unless otherwise indicated
conventional techniques of cell biology, molecular biology, cell culture, immunology and
the like which are in the skill of one in the art. These techniques are fully disclosed in the
25 current literature and reference is made specifically to Sambrook, Fritsch and Maniatis
eds., "Molecular Cloning A Laboratory Manual, 2nd Ed., Cold Springs Harbor Laboratory
Press, 1989); the series Methods of Enzymology (Academic Press, Inc.); Robert M
Horton, "Methods in Molecular Biology, Vol.15: PCR Protocols: Current Methods and
Applications, Ed. B.A> White (Humana Press Inc.) 1993; and Antibodies: A Laboratory
30 Manual, Harlow et al., eds. (1987).

As used in this specification and the claims, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, a stem cell includes a plurality of stem cells.

5 All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications cited herein are hereby incorporated by reference in their entirety in order to more fully describe the state of the art to which this invention pertains.

10

The invention generally described above will be more readily understood by reference to the following examples, which are hereby included merely for the purpose of illustration of certain embodiments of the present invention and are not intended to limit the invention in any way

15

EXPERIMENTAL

Example 1:

- 5 A. *Isolation of human MuSK-R cDNAs* – MuSK-R was isolated by PCR from fetal skeletal muscle cDNA (Marathon cDNA, Invitrogen) using primers flanking the 5' and 3' of the MuSK cDNA. The 5' primer was MuSK 34FN and the 3' primer was MuSK2666R. Cycle 1 – 12 of the PCR reaction was performed with Advantage cDNA Polymerase and Pfu Polymerase. Cycle 13 – 54 of the PRC reaction was performed with
- 10 Pfu Polymerase alone i.e. (WHAT IS Pfu?????) (PLEASE PROVIDE A REFERENCE FOR PCR TECHNIQUE USED. ARE THE CYCLE TIMES STANDARD OR UNIQUE TO YOUR EXPERIMENT – IF UNIQUE WHAT DOES 30'', 1' and 6' STAND FOR cycle 2 – 11:30'' at 94C, 1' at 63C, 6'at 68C)

- 15 The following primers obtained from Life Technologies were used to amplify MuSk cDNA:

MuSK34FN:	gcc tgg att aat cat gag aga gct c	SEQ ID NO: 2
MuSK2666R:	cga ggc ctg tct tca acc tta gac act cac agt tcc ctc gc	SEQ ID NO: 3
MuSK1380F:	cct gtg cca gac tgc cac atc tag ac	SEQ ID NO: 4
20 MuSK1657Rx:	cct gtt aac cct agg tga ggg tta ctg ctg ctg att ctc	SEQ ID NO: 5
MuSK17774R:	ggt taa ccc tat tca atg tta ttc ctt gaa tac tcc ag	SEQ ID NO: 6

- A 2632 base pair fragment was isolated, and the cDNA was cloned into the SrfI site of pCRScript (Stratagene) using standard techniques and sequenced. The wild type
- 25 MuSK-R sequence is illustrated in Figure 2 and corresponds to the sequence of SEQ ID NO: 1.

- (IS THIS ENOUGH DETAIL: STANDARD-COULD ONE OF ORDINARY SKILL IN THE ART WHO OBTAINED THE cDNA FROM MARATHON FOLLOW THE
- 30 TEACHING ABOVE AND ISOLATE THE 2632 FRAGMENT?)

B. *Generation of mutations in the intracellular domain by PCR* - To delete large portions of the intracellular domain of MuSK-R standard PCR procedures were used to introduce stop codons close to the transmembrane domains. The 3' primer MuSK1657R (IS THIS THE SAME AS 1657Rx?) contained the stop codon. The 5' primer MuSK1380F was usually close to a restriction site, so that the PCR product containing the stop codon could be used to replace the wild type (WT) sequence.

The product obtained was amplified and the sequence was designated MuSK-RI. The intracellular domain of WT MuSK (SEQ ID No: 1) is _____ amino acids long. MuSK-RI is illustrated by the deletion of 342 amino acid residues from the 362 amino acid long intracellular domain of WT MuSK. This corresponds to deletion of amino acid residues 537 – 879 in SEQ ID NO: 1. A second product was amplified and this sequence was designated MuSK-RII. MuSK-RII is illustrated by the deletion of 312 amino acid residues from the intracellular domain of WT MuSK. This corresponds to deletion of amino acid residues 557 – 879 in SEQ ID NO: 1. It is believed that both truncations resulted in a deletion of the kinase domain and most of the substrate binding motifs of WT MuSK.

KINASE DOMAIN AND SUBSTRATE BINDING MOTIFS..... THIS IS IMPORTANT WITH RESPECT TO THE DETAILED DESCRIPTION SECTION ON SEQUENCES... IS THE KINASE DOMAIN THE SAME AS THE CATALYTIC DOMAIN? DOES IT INCLUDE ONLY A PART OF THE INTRACELLULAR DOMAIN? IS THERE ONLY ONE KINASE DOMAIN? WHAT IS THE SUBSTRATE BINDING MOTIF?

25

C. *Generation of retroviral vectors containing mutated MuSK-Rs and viral supernatants* - MuSK-RI, MuSK-RII and WT MuSK corresponding to SEQ ID NO: 1, were cloned into the multiple cloning site of the Moloney Murine Leukemia Virus (MoMLV) based retroviral vector pG1a (GTI, Maryland) – (pG1a-mutated sequence –IRES-NGFR). The retroviral vectors were designated pG1aMuSK-I and pG1aMuSK-II. The constructs were cotransfected into 293T cells (OBTAINED FROM WHERE)

30

with an envelope construct pCiGL that permits expression of the Vesicular Stomatitis Virus G-Protein (VSV-G envelope) under the control of the cytomegalovirus (CMV) (PLEASE PROVIDE A REFERENCE _____) I THINK A FIGURE WITH THE VECTOR CONSTRUCT SHOULD BE USED AS WELL).

5

Also cotransfected into HEK 293T cells was the packaging construct pCiGP (encoding MoMLV gag-pol _____ (AND OBTAINED FROM WHERE _____) using the CaCl₂ technique (Clontech).

10

Using standard techniques as described in (PLEASE PROVIDE REFERENCE _____) viral supernatants were collected 24, 48, and 72 hours after transfection. The viral supernatants were used to transduce the packaging cell line ProPak-A-6 (PPA-6) (Systemix, Inc.). (REFERENCE _____) Supernatants were collected on day 2, 3 and 4 after transduction. The PPA-6 cells were sorted by either bead selection (REFERENCE _____) or FACS sorting (REFERENCE _____) for marker gene expression. The supernatants of the sorted PPA-6 cells contained recombinant virus that has an amphotropic envelope and were used to transduce non-murine cells.

15

20 (AGAIN I AM CONCERNED THAT INFORMATION IS MISSING ON THE TECHNIQUES, VECTORS ETC USED. COULD ONE SKILLED IN THE ART FOLLOW THE ABOVE DESCRIPTION AND COME OUT WITH YOUR RESULTS.

D. Tissue culture and cell lines - The following cell lines and primary cells were used:

25

(a) human T cell line, CEMSS (REFERENCE _____) (b) human embryonic kidney cells 293T (293T), and (c) PPA-6, a human amphotropic packaging cell line which is a derivative of 293T cells. It expresses (WHAT EXPRESSES?) the MLV amphotropic envelope and MLV gag/pol under the control of a CMV promoter (REFERENCE _____).

30

Human primary T cells were obtained by isolating the mononuclear cell fraction from human blood using Ficoll (Lymphoprep - IS THIS A COMPANY or TECHIQUE??? DO YOU HAVE A REFERENCE) gradient centrifugation. Human blood (buffycoats WHAT IS THIS)
5 _____) obtained from the Stanford Bloodbank were diluted 1:1 with PBS (WHAT IS THIS) . In 50 ml tubes, 15 ml blood were overlaid onto 21 ml of Lymphoprep. The gradient was spun for 30 minutes at 1700 rpm at room temperature. Peripheral blood mononuclear cells (PBMCs) were collected from the interface of the Lymphoprep gradient. The resulting PBMCs were
10 further purified on a second Ficoll gradient to remove remaining blood cells. The PBMCs were incubated for 1 hour in a tissue culture flask (CONDITIONS -TEMPERATURE ETC) allowing adherent cells such as macrophages to attach to the tissue culture flask. Non-adherent cells (T/B/NK cells) were used to purify CD4⁺ T cells. Cells (2×10^8) were incubated with 300 μ l α -CD4 antibody
15 for 1 hour at 4°C. The cells were washed 3 times with PBS and incubated with 1×10^8 magnetic beads (Dynal, Oslo) for 1 hour at 4°C. The CD4⁺ cells that bound the CD4-antibodies and magnetic beads were positively selected by incubating for 10 minutes on a Dynal magnet. After removing the unbound cells, the remaining cells were taken off the magnet and put into culture. Usually the beads remained for up to 10 days on the cells.,
20 CD34+ cells were isolated from G-CSF mobilized peripheral blood (MPB) using Isolex 300SA or 300I (Baxter, IL) (Systemix, CA). The cells were approximately 80 – 90% pure.

Cells were cultured in DMEM, 10% FBS, 1% sodium pyruvate, 1% L-glutamine
25 (293 T, PPA-6, CEMSS); Iscoves medium 10% FBS, 1% L-glutamine, (human T cells), 1 % ITS (insulin/transferrin/sodium selenite, 0.5 mg/ml stock), 1% MEM vitamins. Human T cells were stimulated every 10-12 days with 1-2 μ g/ml PHA (phythemaglutinin), irradiated feeder cells and 40 U/ml Il-2. Irradiated feeder cells were generated as follows: PBMC were isolated as described above and irradiated at 3500 rads. The cells were

mixed at a 10:1 ratio with the B cell lymphoma JY (REFERENCE _____)
that had been irradiated at 6000 rads.

To obtain cells in a resting state, 4-5 days after stimulation T cells were
5 transferred into fresh medium that contained 20 U/ml IL-2. CD34⁺ cells were grown in X
vivo 15, 50 ng/ml thrombopoietin (TPO), 100 ng/ml flt-3 ligand (FL), 100 ng/ml steel
factor (SF).

E. *Transduction of PPA-6, human T cell lines, primary T cells and CD34⁺ cells* - 10⁶
10 cells/ml from step (D) were transduced with 1 ml of viral supernatant, that had been
either generated from 293 T cells or PPA-6 cells, by spinoculation with 4 mg/ml
protamine sulfate. Spinoculation was done at 37 C for 3 hrs at 3000 rpm (CD34⁺ cells) on
one day or at 2300 rpm (human T cells) on two subsequent days. (PLEASE PROVIDE
REFERENCE _____) Human T cells and CD34⁺ cells were
15 activated for 2 days prior to the transduction procedure with PHA/IL-2 or TPO/FL/SF
respectively. (WHAT IS PHA?? PLEASE PROVIDE CONCENTRATIONS
_____)

F. *FACS analysis and FACS sorting of cells that express MuSK-I and MuSK-II* - FACS
20 analysis was done on a FACScan (Becton Dickinson). The following antibodies and
reagents were used for staining. CD4-FITC (Caltag), α CD34-APC (Becton Dickinson),
Thy-1-PE, propidium iodide (PI), α -Flag biotinylated (Sigma), goat α -mouse Fab
(Calbiochem), goat α -mouse IgG2a-PE (Calbiochem), streptavidin-PE (Calbiochem)
(OTHER ANTIBODIES _____) and magnetic beads
25 (Dynal, Oslo). Staining was done in PBS/2% FBS for 20 minutes to 60 minutes at 4C. To
avoid binding of antibodies to F_c-receptors 250 μ g/ml normal rabbit serum was added.

To sort cells for the marker gene (MuSK-RI and MuSK-RII), cells were stained
with a fluorophore conjugated antibody (FITC, PE) or an unconjugated antibody was
30 used (in which case a secondary fluorophore couples antibody that can recognize the

primary unconjugated was used. The cells were also stained with PI to distinguish between dead and live cells.

(I DO NOT THINK ENOUGH DETAIL IS PROVIDED HERE??)

5

To isolated cells by bead selection, cells were stained with a primary antibody that recognizes the molecule of interest **(THIS IS NOT SUFFICIENT --- THE SPECIFIC MOLECULE OF INTEREST AND THE PRIMARY ANTIBODY MUST BE IDENTIFIED)** The antibody coated cells are incubated with magnetic beads that are coupled to antibodies that can recognize the primary antibody.

10

Finally the cells with the marker gene (EGFR-I and EGFR-II) that had bound up a marker gene antibody and magnetic beads are positively selected by incubating them for 10 minutes on a Dynal magnet. **(FOR HOW LONG NEED DETAILS!!!)**

15

The unbound cells were removed by techniques well known in the art **(REFERENCE _____)** and the remaining cells were taken off the magnet and put into culture **(WHAT CULTURE _____)** The beads remain for about 10 days on the cells. **THEN WHAT?**

20

Figure 3 illustrates the expression of the WT MuSK-R, MuSK-RI, and MuSK-RII on primary human T cells and CD34⁺ cells after transduction of these cells with supernatants that were generated from PPA-6 cells. In addition, primary human T cells were sorted using a FACStar III. The cells were enriched from _____% to about 95 %.

25

(PLEASE PROVIDE FIGURE)

IT IS CLAIMED:

1. A method of using a muscle specific tyrosine kinase receptor molecule (MuSK-R) or a mutant receptor molecule thereof as a selectable marker in a mammalian cell comprising,
 - a) incorporating a nucleic acid sequence encoding the coding region of a MuSK-R or a mutated MuSK-R into a population of mammalian cells;
 - b) allowing expression of the nucleic acid sequence in said cells wherein said cells are not capable of signal transduction; and
 - c) selecting the cells expressing the MuSK-R or mutant MuSK-R.
2. The method according to claim 1, wherein the mutated MuSK-R molecule is a MuSK-R sequence having at least 150 amino acids deleted from the intracellular domain.
3. The method according to claim 2, wherein the mutated MuSK-R molecule is a MuSK-R sequence having at least 300 amino acids deleted from the intracellular domain.
4. The method according to claim 1, wherein the mutated MuSK-R molecule is a MuSK-R sequence having the kinase catalytic site deleted.
5. The method according to claim 2, wherein the mutated MuSK-R is MuSK-RI.
6. The method according to claim 2, wherein the mutated MuSK-R is MuSK-RII.
7. The method according to claim 1, wherein the nucleic acid sequence encoding the mutated MuSK is introduced into the mammalian cell by a vector.
8. The method according to claim 7, wherein the vector is a retroviral vector.
9. The method according to claim 1, wherein the mammalian cells are human cells.

10. The method according to claim 1 or 9, wherein the cells are hematopoietic cells.
11. The method according to claim 10, wherein the cells are hematopoietic stem cells.
- 5 12. The method according to claim 10, wherein the cells are hematopoietic cells are T-cells.
13. The method according to claim 1, wherein the nucleic acid encoding the MuSK-R or mutant MuSK-R is introduced in combination with a second nucleic acid encoding
10 sequence wherein the second sequence encodes a protein of interest.
14. The method according to claim 1, further comprising isolating the selected cells expressing the MuSK-R or mutated MuSK-R.
- 15 15. A method of selecting genetically modified mammalian cells comprising,
a) incorporating a nucleic acid encoding a muscle specific tyrosine kinase
receptor (MuSK-R) or mutated MuSK-R into a population of mammalian
cells;
b) introducing a heterologous DNA sequence which encodes a protein of interest
20 into the population of mammalian cells;
c) allowing expression of the MuSK-R or mutated MuSK-R in said cells; and
d) selecting genetically modified mammalian cells expressing said MuSK-R or
mutated MuSK-R.
- 25 16. The method according to claim 15, wherein the mutated MuSK-R molecule is a MuSK-R sequence having at least 150 amino acids deleted from the intracellular domain.
17. The method according to claim 16, wherein the mutated MuSK-R is selected from MuSK-RI and MuSK-RII.

30

18. The method according to claim 15, wherein said heterologous DNA sequence encoding the protein of interest is introduced on the same vector as the MuSK-R or mutated MuSK-R.

- 5 19. A method for the immunoselection of transduced mammalian cells comprising,
- a) transducing cells with a nucleic acid sequence encoding a muscle specific tyrosine kinase molecule (MuSK-R) or a mutated MuSK-R molecule;
 - b) incubating the cells with a marked antibody which recognizes and binds specifically to the MuSK-R or mutated MuSK-R; and
 - 10 c) identifying the marked transduced cells.

20. The method according to claim 19, wherein the cells are human cells.

21. The method according to claim 19, wherein the cells are hematopoietic cells.

15

22. The method according to claim 19, wherein the cells are transduced by a retroviral vector selected from _____.

23. The method according to claim 19, further comprising separating the identified
20 marked transduced cells from non-marked cells.

24. The method according to claim 19, further comprising expanding the marked transduced cells.

- 25 25. A method of identifying mammalian cells expressing a protein of interest, comprising
- a) introducing into a population of mammalian cells a nucleic acid encoding a muscle specific tyrosine kinase molecule (MuSK-R) or a mutated MuSK-R molecule, wherein said MuSK-R or mutant MuSK-R can not effect signal
30 transduction, and introducing a nucleic acid comprising a DNA sequence encoding a protein of interest into said population;

- b) culturing the mammalian cells under conditions which favor growth and expansion of said cells; and
- c) selecting cells which express MuSK-R or mutated MuSK-R thereby obtaining cells which express the protein of interest.

5

ABSTRACT

5 The present invention concerns a method of using a muscle specific tyrosine kinase receptor molecule (MuSK-R) or a mutated MuSK-R thereof as a selectable marker in mammalian cells. Particularly preferred are MuSK-R molecules incapable of signal transduction and preferably molecules wherein the intracellular domain has been modified by deletion of the signaling region. The invention also relates to a method of selecting genetically modified mammalian cells by using a MuSK-R or a mutated MuSK-R thereof as a selectable marker. Further a method for the immunoselection of transduced
10 mammalian cells is disclosed comprising identifying the transduced cells by incubation of the cells with a marked antibody which recognizes and binds specifically to a MuSK-R or mutated MuSK-R thereof.